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NEW APPROACHES TO HEPATITIS A VACCINE DEVELOPMENT

ANNUAL REPORT

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Hepatitis A virus (HAV) has historically been an important cause of morbidity among U.S. soldiers in the field. Work under this contract is directed at the development of a safe, inexpensive and effective hepatitis A vaccine for use in military personnel. Two main research approaches are under investigation. First, we are examining synthetic oligopeptides representing the suspected surface structures of HAV. Octapeptides have been synthesized on polyethelene pins and probed with polyclonal and monoclonal antibodies in an effort to identify antigenic and potentially immunogenic sequences. We have also determined the ability of poliovirus-HAV chimeric viruses to elicit neutralizing antibodie to HAV. The second approach involves the construction of an infectious cDNA construct derived from a virulent but cell culture-adapted variant of HAV. Genetic maninulation of such a construct will allow alternative approaches to the development of attenuated vaccine candidates. A third aim is to develop PCR-related technology permitting the identification of specific strains of HAV, as this will facilitate clinical studies of HAV vaccines and future military disease surveillance activities.

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FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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INTRODUCTION

Commercial interest in development of formalin-inactivated HAV vaccines has resulted in products that appear safe and reasonably immunogenic in phase I and early phase II clinical trials (for a recent review, see Siegl and Lemon, 1990). However, there are several unresolved questions concerning the use of such vaccines. The magnitude and speed of the neutralizing antibody response to inactivated HAV appears directly related to the quantity of inactivated viral antigen included in each dose. Vaccines containing less than 200-400 ng purified antigen appear to require multiple administrations in order to elicit protective levels of immunity. Multiple-dose schedules, with late booster doses given at 6 months in several current clinical trials, may prove inconvenient for use in military populations. Perhaps of greater practical significance, the future cost of such vaccines is unknown but likely to be quite high. This is due to the comparatively poor in vitro yields of antigen obtained with current vaccine virus strains, and the purification procedures required for production of an acceptable, modern vaccine. High costs will probably prohibit the universal use of inactivated vaccines among U.S. military forces. Lastly, HAV is exceptionally stable to thermal inactivation and extremely prone to aggregation. Thus, the ability to completely inactivate production-sized lots of cell culture-produced HAV remains a continuing concern that will only be resolved by more extensive clinical investigation of inactivated vaccines. Because of the uncertainties concerning future use of inactivated HAV vaccines, work under this contract includes efforts to develop alternative approaches to HAV vaccines.

In previous work, we have mapped an immunodominant neutralization antigenic site on the surface of the HAV capsid by analysis of murine monoclonal antibody-resistant neutralization escape variants of HAV (Ping et al., 1988; Cox et al., 1990), and by characterizing the competition between such monoclonal antibodies for binding to the virus capsid (Stapleton et al., 1987). These studies indicate that the βB-βC loops of capsid proteins VP3 and VP1 contribute to an immunogenic structure on the virus surface that dominates in the human immune response (Ping et al., 1988; Day et al., 1990a). Although, this site is largely conformationally defined, we have reasoned that short oligopeptide sequences representative of the relevant regions of VP3 and VP1 should be both antigenic and potentially immunogenic with respect to HAV neutralizing activity. This has been shown to be the case with peptides representative of antigenic sites in two other picornaviruses that are closely related to HAV, type 1 poliovirus (Chow et al., 1985) and human rhinovirus 14 (Francis et al., 1989), as well as the more unique foot-and-mouth virus (FMDV) (Bittle et al., 1982). Peptide immunogens are highly stable reagents; they are potentially very inexpensive and extremely safe inasmuch as they are chemically defined. As we have previously shown that only low levels of neutralizing antibody are required for protection against hepatitis A (Stapleton et al., 1985), we have postulated that peptide immunogens may have practical application to the prevention of hepatitis A.

The experimental approach taken has been the synthesis of octapeptides representing the βB-βC loops of capsid proteins VP3 and VP1 on polyethylene pins, and the probing of such peptides with monoclonal and

polyclonal anti-HAV antibodies in peptide ELISAs (PEPSCAN) (Geysen et al., 1984, 1987). However, an alternative approach which we have recently begun to pursue in collaboration with Prof. J. Almond of the University of Reading, Reading, Berks., U.K. and Dr. P. Minor of the National Institute of Biologics Standardization and Control, Potters Bar, Herts., U.K., has been the insertion of appropriate HAV peptide sequences into an antigenic loop of capsid protein VP1 of the Sabin type 1 virus. These chimeric picornaviruses have been constructed using an infectious poliovirus cDNA clone which contains a mutagenesis cassette in the region encoding for VP1 (Burke et al., 1989). Other poliovirus chimeras have proven to be successful vectors for immunogenic peptides. HAV/poliovirus chimeras have the potential of presenting HAV peptides in a conformationally constrained manner, and we have been encouraged by preliminary success with such chimeric viruses.

In addition to exploring these approaches to subunit HAV vaccines, we have continued efforts to develop an infectious HAV cDNA clone derived from a virulent but highly cell culture-adapted virus (p16 HM175). This work follows on from that supported under a previous contract with the U.S. Army Medical Research and Development Command (DAMD17-85-C-5272). Commercial attempts to develop an attenuated HAV vaccine have focused on the attenuation of HAV that follows adaptation of virus to cell culture (for a review, see Lemon, 1985; Siegl and Lemon, 1990). Such viruses appear to replicate poorly in the primate liver, and have very poor immunogenicity in man. There is thus a need for novel approaches to selecting attenuated HAV vaccine candidate strains.

Development of an infectious, virulent construct would be useful in further characterizing the molecular basis of adaptation of virus to growth in cell culture, as well as attenuation of HAV. Furthermore, as deletions within the 5' nontranslated region (5' NTR) of virulent, Mahoney type 1 poliovirus have been shown by Iizuka et al. (1989) to result in significant attenuation, we would propose to use an infectious, virulent cDNA HAV clone to generate similar 5' NTR deletion mutants of HAV. A genomic-length cDNA clone derived from p16 HM175 (Jansen et al., 1988) has been constructed within the transcriptional vector pGEM-3. Although this construct is not viable in transfection assays, either as RNA or DNA, transfection experiments with chimeric molecules constructed with a viable, but attenuated full-length clone (pHAV/7, Cohen et al., 1988) suggests that a lethal mutation exists between bases 4977 and 7003 of the p16 clone. This region is now being re-sequenced and replaced with cDNA from alternative p16 and wt HAV cDNA clones.

Lastly, a major part of the present contract work includes efforts to characterize the molecular epidemiology of HAV. This aim has been approached by development of a simplified polymerase chain reaction (PCR)-based method of viral genome analysis that is suitable for use in clinical trials of HAV vaccines and for future hepatitis A disease surveillance activities. Using this antigen-capture/polymerase chain reaction (AC/PCR) method (Jansen et al., 1990a), we have characterized 47 fecal strains and cell culture isolates of HAV and have gained valuable new information concerning the molecular epidemiology of this virus. With minor changes, this method may also be applicable to surveillance of hepatitis E virus (HEV) or other viral pathogens of military significance.

RESEARCH PROGRESS

Antigenicity of HAV peptides

The specific approach we have taken to mapping antigenic peptides of HAV involves the synthesis of nested octapeptides on polyethylene pins. These peptides, overlapping each other by 7 residues, are probed in enzyme-linked immunosorbent assays (ELISA) which assess the binding of immunoglobulins to specific peptide-bearing pins (PEPSCAN) (Geysen et al., 1984, 1987). This is an approach that we have successfully applied to mapping the antigenic structure of hepatitis delta virus (Wang et al., 1990). During the past year we have synthesized octapeptides representative of the putative βB-βC loops of VP3 (residues 3050-3091)[1] and VP1 (residues 1080-1130) of HAV. These regions of HAV VP3 and VP1 contain mutations conferring neutralization escape against a variety of murine and human monoclonal antibodies (Ping et al., 1988; Cox et al., 1990). The validity of the peptide synthesis was documented by synthesis of antigenically active oligopeptides reactive with known antipeptide antisera.

HAV octapeptides have been probed with a variety of polyclonal and monoclonal antisera. In general, only very low level reactivity has been noted with polyclonal anti-HAV antisera in ELISA tests. Screening with a high titer human polyclonal antiserum (JC, diluted 1:1000) suggested possible reactivity with octapeptides spanning residues 3067-3079 (Figure 1). This activity was minimal, resulting in an O.D. value less than 0.2 which is normally accepted as evidence of significant reactivity in our laboratory (Wang et al., 1990). However, this minimal signal was somewhat reduced by substitution of aspartic acid-3070 with histidine (particularly with the peptides SDSVGQQI and DSVGQQIK) (data not shown), as might be expected given previous analysis of escape mutants (Ping et al., 1988). This human antiserum did not react with VP1 peptides.

A polyclonal rabbit anti-HAV antiserum demonstrated greater reactivity against the octapeptides (Figure 2). This rabbit serum (A/S 8.9.88, a generous gift from Dr. D. Sangar, Wellcome Biotech, Beckenham, Kent, U.K.) was raised against purified cell culture-derived HAV and neutralizes HAV at dilutions up to 1:10,000. In PEPSCAN assays at a dilution of 1:1000, peptides representing residues 3050-3061 were reactive, as were peptides spanning residues 1109-1122. The validity of this octapeptide scan is suggested by the fact that the latter region is proximate to two residues known to be the site of neutralization escape mutations (1102 and 1114). However, the possibility that this serum contains antibodies against denatured or partially denatured capsid proteins cannot be excluded. In addition, we recognize that nonspecificity of reactions is a problem in testing polyclonal sera by PEPSCAN, even at serum dilutions of 1:1000 or greater, and we consider

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[1] By convention, HAV capsid protein amino acid residues are designated by four-digit numbers, the first of which indicates the specific capsid protein, followed by three digits indicating residue number. Assignment of HAV residues to specific loop structures is based upon alignments, created by Dr. A. Palmenberg of the University of Wisconsin, of HAV capsid proteins with capsid proteins of viruses with known crystal structures.

these results to be preliminary. Confirmation is being sought by testing against peptides synthesized in bulk by conventional solid-phase methods.

We are currently screening a panel of 24 neutralizing anti-HAV monoclonal antibodies against VP3 and VP1 nested octapeptides. Antibodes evaluated thus far include K24F2, 10.09, K32F2, and AE8. No reactivity has yet been found with monoclonal antibodies against either VP3 or VP1 octapeptides, even though it is known by mutant analysis that these antibodies recognize epitopes which are comprised, in part, of amino acid residues 3070 or 1102. Although these data suggest that the relevant epitopes of HAV are highly conformational, these studies remain in progress (see "Conclusions" below).

Analysis of HAV/poliovirus chimeras

During the past year we have initiated a collaboration with Prof. J. Almond of the University of Reading, Reading, Berks., U.K. and Dr. P. Minor of the National Institute of Biological Standardization and Control (NIBSC), Potters Bar, Herts., U.K. in order to evaluate chimeric picornaviruses in which potential HAV epitopes have replaced part of the antigenic VP1 βB-βC loop of Sabin type 1 poliovirus. The availability of an infectious cDNA clone of the Sabin type 1 virus containing a mutagenesis cassette in the region encoding the VP1 βB-βC loop (Burke et al., 1989) has facilitated the construction of HAV/poliovirus chimeras. A total of 12 viable poliovirus/HAV chimeras expressing a variety of HAV sequences have thus far been generated (see Table 1), and antisera have been raised against these viruses in small animals at NIBSC. We have carried out HAV neutralization tests with anti-chimera antisera, and have assessed the HAV antigenicity of chimeric viruses.

The results of HAV neutralization assays with anti-chimera antisera are summarized in Table 1. Neutralization of HAV was assessed in radioimmunofocus-reduction assays. At serum dilutions of 1:10, significant neutralizing activity was present in 2 of 7 immunized rabbits and in a single mouse serum pool raised to the HAV/poliovirus chimera S1/H15, while 1 of 4 rabbits developed neutralizing antibodies following immunization with chimera S1/H2. Positive results were reproducible, and rabbit prebleeds were devoid of neutralizing activity in each case. The mouse anti-S1/H15 pool had the highest anti-HAV titer (approximately 1:100). A more detailed summary of anti-S1/H15 sera is shown in Table 2. Although several anti-S1/H15 sera neutralized HAV, none were capable of immunoprecipitating ^{3}H-uridine labelled HAV.

The S1/H15 chimera contains residues representing an amino terminal domain of HAV VP1. A synthetic peptide with similar sequence was reported previously by Emini et al. (1985) to be immunogenic and capable of eliciting neutralizing anti-HAV activity (Figure 1). An analogous poliovirus peptide has similarly been reported to induce neutralizing antibody to poliovirus by Chow et al. (1985), despite the fact that this VP1 domain has an internal position within the native poliovirus capsid (Hogle et al, 1985). However, Frick and Hogle (1990) have recently demonstrated that the amino terminus of VP1 of poliovirus is externalized during the conformational shift to 135 S particles ("A" particles) that accompanies cell attachment and early steps of penetration of poliovirus. This sequence of events provides a potential explanation for the

neutralizing activity of antisera directed against the VP1 amino terminus of HAV, and may explain why anti-S1/H15 antisera which neutralize HAV infectivity fail to immunoprecipitate native virus.

Table 1. Anti-HAV neutralizing activity of antiserum raised to PV1/HAV chimeric viruses

Chimera	HAV residues in VP1 βB-βC loop	Positive antisera/Number tested* Rabbit	Guinea Pig	Mouse
S1/H15	1013-1024	2/7	nd	1/1**
S1/H1	1015-1020	nd	0/3	0/3
S1/H11	1029-1041	nd	0/2	0/3
S1/H14	1070-1081	nd	0/1	nd
S1/H10	1099-1107	0/5	0/3	0/3
S1/H2	1101-1108	1/4	0/4	0/3
S1/H3	1150-1155	nd	0/3	0/3
S1/H5	1192-1197	nd	0/3	0/3
S1/H6	1217-1224	nd	0/2	0/3
S1/H13	2047-2055	nd	0/1	nd
S1/H16	3067-3079	0/5	nd	nd

*positive defined as ¢80% HAV RFU reduction at 1:5-1:10 diln
**pool of 3 mouse sera

Sera from two guinea pigs immunized with a KLH-conjugated peptide corresponding to residues 11 to 32 of VP1 of HAV (peptide 639c, provided by Dr. D. Sangar of Wellcome Biotech) (Figure 3), although reactive with this peptide at dilutions greater than 10^5 in solid-phase peptide ELISA, failed to neutralize HAV (Table 2). In contrast, only the M18 mouse serum pool and neither of the rabbit antisera raised against the S1/H15 chimeric virus reacted with this peptide in ELISA, despite the fact that it contains all of the HAV residues expressed by the chimera. The anti-peptide ELISA titer of the M18 serum (1:100) was the same as its HAV 50% neutralization endpoint titer (also 1:100). These data suggest that inclusion of the HAV sequence within the VP1 loop of the poliovirus chimera has placed favorable conformational constraints on the peptide, resulting in significant although only partial HAV immunogenicity of the chimera.

More recently, we have assessed the anti-peptide activities of these sera in PEPSCAN assays against octapeptides representative of the chimera VP1 βB-βC loop and the HAV amino terminal VP1 domain. These studies document an appropriate but low titer anti-peptide response in the rabbits developing HAV neutralizing activity, and thus demonstrate that PEPSCAN may have significantly greater sensitivity than conventional microtiter peptide ELISA. However, PEPSCAN studies confirm the much greater anti-peptide activity of anti-peptide compared with anti-chimera sera, supporting the notion that the chimera induces anti-HAV activity by presenting the peptide sequence in a unique conformation.

Table 2. Anti-chimera and anti-HAV activity of PV1/H15 and peptide 639c antisera.

Serum	Date	Doses	$\log_{10}$ S1/H15 RIP	$\log_{10}$ HAV RIP	$\log_{10}$ HAV neut
R81*	1. 8.89	4	2.6	≥1	1
R82	1. 8.89	4	2.8	≥1	1
R93	15.12.89	3	3.8	≥1	≥1
R94	15.12.89	3	4.0	≥1	≥1
R100	10. 1.90	2	4.3	≥1	≥1
R101	10. 1.90	2	4.4	≥1	≥1
R102	10. 1.90	2	3.9	≥1	≥1
M18 pool**			3.3	≥1	2
gp639c/7	4. 4.89		4.3	≥1	≥1
gp639c/8	4. 4.89		4.9	≥1	≥1

Notes: RIP (radioimmunoprecipitation) carried out with ^{3}H-uridine labelled virus; titers defined as 50% RIP or 50% neutralization endpoints. gp639c: guinea pig antisera against HAV peptide 639c, HAV residues 1011-1032 coupled to KLH (gift of Dr. D. Sangar). (*rabbit sera, **mouse serum pool).

Because the anti-peptide 639c sera reacted strongly with the chimeric S1/H15 virus (Table 2) in both radioimmunoprecipitation (¢1:10,000) and virus neutralization assays (¢1:5000), the chimeric virus must also present the HAV sequence in the conformation assumed by the peptide when conjugated to KLH. Thus, the chimeric loop region exists in at least two conformations, one which is similar to that assumed by the peptide and another which more closely mimics the conformation of these residues in the HAV structure. Indirectly, these data indicate that the VP1 amino terminal neutralization epitope is conformationally defined.

The extent to which the S1/H15 chimeric virus is susceptible to neutralization or precipitation with anti-peptide antisera appears to be unique among all poliovirus chimeras studied to date (including intertypic poliovirus chimeras and FMDV/poliovirus chimeras), and is reminiscent of the neutralization of FMDV with anti-peptide antisera (Bittle et al., 1982). Crystallographic studies have suggested that the FMDV VP1 G- H loop, the target loop for neutralization of FMDV with anti-peptide sera, is a highly disordered structure (Acharya et al., 1989). This feature probably accounts for the unique antigenic and immunogenic activity of FMDV VP1 peptides. The high-titered neutralizing activity of anti-peptide antisera against the S1/H15 virus thus suggests that the inclusion of HAV residues has uniquely mobilized the VP1 βB-βC loop in the chimeric virus. Significant disorder in this chimeric VP1 loop may account in part for the HAV immunogenicity of the chimera, and is consistent with the serologic evidence for alternative conformations described above.

Anti-S1/H15 sera which lack HAV neutralizing activity have been tested by PEPSCAN to determine whether the absence of a response against HAV was due to a general failure to induce antibodies against the chimeric

βB-βC loop. Thus far, these studies indicate that all animals immunized with S1/H15 have developed antibodies against HAV residues included in the loop, although only some animals have raised antibodies capable of neutralizing HAV.

One of 4 rabbits immunized with the S1/H2 chimera (HAV residues 1101-1108) developed significant anti-HAV neutralizing antibodies. The HAV domain included in the chimeric virus in this case includes a residue known to be the site of a neutralization escape mutation (residue 1102), and the chimera most likely represents a VP1 βB-βC loop replacement (which results in immunogenic inter-typic poliovirus chimeras). However, although seroconversion in a single animal is encouraging, other rabbit, guinea pig and mouse sera were negative for HAV neutralizing activity (Table 1). We are in the process of constructing additional chimeras in which the inserted HAV sequence will be extended to include residue 1114 of HAV, which we have determined by site-directed mutagenesis of an infectious cDNA clone to be a site of neutralization escape mutation (Cox et al., 1990). In addition, PEPSCAN analysis of the βB-βC loop of S1/H2 (residues 1087-1108) is being pursued as described above for the S1/H15 chimera.

We have been very encouraged by these preliminary results with HAV/poliovirus chimeras, and plan further studies in the next year of this contract (see "Conclusions" below). These studies will include PEPSCAN analysis of the amino terminus of VP1 using polyclonal primate anti-HAV positive sera, and analysis of the immunogenicity of heat-denatured chimeras (particularly S1/H15 and S1/H2).

Molecular basis of HAV cell-culture adaptation and attenuation

Infectious cDNA construct derived from virulent HAV We have continued efforts to develop an infectious cDNA clone derived from a virulent but highly cell culture-adapted HAV variant (p16 HM175) (Jansen et al., 1988). Such a construct will be useful in further characterizing the molecular basis of adaptation of virus to growth in cell culture, as well as attenuation of HAV. A genomic-length cDNA clone derived from p16 HM175 has been assembled within the transcriptional vector pGEM-3. Details of this construction may be found in the final report of our previous contract with the U.S. Army Research and Development Command (DAMD17-85-C-5272). The full-length p16 construct has not been found to be viable in transfection assays in BS-C-1 cells, either as RNA or DNA. However, analysis of chimeric molecules constructed with the viable, attenuated pHAV/7 clone (Cohen et al., 1988) suggests that there is a lethal mutation between bases 4977 and 7003 of the p16 clone (Figure 4). This region of the clone is now being re-sequenced and replaced with cDNA from alternative p16 and wt clones.

Role of 5' NTR mutations in cell culture adapation Previous comparisons of mutations present in cell culture-adapted HM175 virus variants have suggested that mutations within the 5' NTR may play an important role in altering the host range of the virus and in determining attenuation (Jansen et al., 1988). To assess the role of mutations within the 5' NTR in determining the replication competence of cell culture-adapted HAV in cell culture, we constructed several chimeric viruses in which regions of the 5' NTR of pHAV/7 have been replaced with sequences

derived from wt or the cell culture-adapted but virulent p16 HM175. The capacity for growth of these viruses in BS-C-1 cells has been assessed by determining focus-size ("plaque" size) in radioimmunofocus assays. In contrast to previous reports by Purcell and coworkers which have indicated that mutations in proteins 2B/2C have a central role in determining cell-culture adaptation, these studies suggest that mutations within the 5' NTR strongly influence the ability of virus to replicate in BS-C-1 cells (Day et al., 1990b). Small plaque variants of HAV were obtained when the p35 5' NTR was replaced with wt 5' NTR or the p16 5' NTR up to but not including the mutation at base 687. Total replacement of the p35 5' NTR with p16 5' NTR resulted in large plaque virus, suggesting that the mutation at 687 plays an important role in determining the ability of the virus to replicate in BS-C-1 cells (Figure 5). Impaired replication of 5' NTR chimeras was not associated with reduced translational ability in rabbit reticulocyte lysates. This does not rule out a role for these mutations in translation, however, as they may determine the ability of the HAV 5' NTR to interact with host cell-specific proteins involved in virus translation. We plan to determine whether the small-plaque variants are temperature sensitive.

Mutations associated with cytopathic HAV Two cytopathic variants of HAV have been clonally isolated from persistently infected BS-C-1 cells; both isolates have been partially sequenced. One of these rapidly-replicating, highly cell culture-adapted variants (HM175/43c) was found to be a spontaneous neutralization escape variant, with an Asp to Ala substitution at residue 3070. As described in the final annual report of our previous contract with the U.S. Army Research and Development Command (DAMD17-85-C-5272), this variant originated in the absence of any antibody pressure, and predominated in persistent infection of BS-C-1 cells with HM175 virus, suggesting that this mutation may confer a replication advantage under these growth conditions. The second cytopathic variant (HM175/18f) has a normal antigenic phenotype, and rapidly replaces the antigenic variant when harvests from persistently infected cells are serially passaged in FRhK-4 cells. This variant has exceptional in vitro growth properties, and has been supplied to Dr. Leonard Binn of the Department of Virology, Walter Reed Army Institute of Research, for use in HAV neutralization assays. Both cytopathic variants share identical but otherwise unique mutations in the 5' NTR. Sequence analysis of P2 and P3 genomic regions is currently in progress.

Molecular Epidemiology of HAV

We have established an extensive data base containing partial genomic sequences of 47 HAV fecal strains or HAV cell culture isolates. We have partially sequenced the VP3 and VP1/2A encoding regions of each virus (total of 325 bases from each virus), using an antigen-capture/ polymerase chain reaction (AC/PCR) method (Jansen et al., 1990a). Development of this method was described in detail in the final annual report of our former contract with the U.S. Army Research and Development Command (DAMD17-85-C-5272). In brief, immunoaffinity capture of virus, heat denaturation of virus, synthesis of viral cDNA, and amplification of cDNA by a polymerase chain reaction are carried out sequentially in a single reaction vessel. This approach simplifies sample preparation and enhances the specificity of conventional PCR. The ability of AC/PCR to rapidly generate sequence data directly from virus present in fecal

specimens represents a significant advantage. Thus far, analysis of partial genomic sequences from 47 virus strains has demonstrated remarkable conservation among most human HAV strains, but has also revealed hitherto unsuspected genetic diversity among a small number of human isolates (Jansen et al., 1990b). These findings are summarized in Figure 6. Results to date support the existence of 3 distinct HAV genotypes, which we have arbitrarily defined as strains differing at more than 15% of bases sequenced, following a previous definition of poliovirus genotypes (Rico-Hesse et al., 1987).

It was surprising to find that 7 cell culture-adapted virus isolates were identical in sequence to the MBB strain (not shown). We suspect that many of these isolates represent contamination of cell lines with MBB virus, as this virus, isolated in Prof. Deinhardt's laboratory in Munich, was one of the first HAV cell culture isolates and has apparently been carried as a reference strain in many laboratories. We know this is the case with a "KMW-1" cell culture isolate with sequence identical to MBB, as sequence derived by AC/PCR from the original KMW-1 fecal specimen was unique and had only 92.6% identity with MBB. Similar evidence indicates that a cell culture-adapted "S85-1" isolate is an HM175 strain contaminant. Such contamination appears to have occurred in a number of laboratories engaged in the isolation HAV in cell cultures, indicating that firm conclusions concerning the epidemiologic relatedness of strains sharing identical sequences may only be based on sequences derived directly from virus present in clinical marterials.

Of note, cell-culture adapted CLF strain HAV (obtained from Dr. G. Siegl of the University of Bern, Bern, Switzerland) was identical in sequence to the MBB strain within both genomic regions. Although we suspect that CLF may also be an MBB contaminant, we have been unable thus far to confirm this as our PCR primers do not amplify HAV sequences present in the original CLF fecal sample. This strain has been used for the production of an inactivated HAV vaccine which has entered human clinical trials.

As might be expected, viruses present in fecal samples collected from three American soldiers (GR-1, GR-7 and GR-CL) involved in the 1982 Grafenwohr outbreak of hepatitis A in the Federal Republic of Germany (see relevant EPICON report) shared a common nucleotide sequence. Similarly, fecal specimens collected from four cases of endemic hepatitis A occurring in central Greece during 1983 (AG11, AG5978, AG6014 and AG6084) contained viruses that were identical or differed at only one base position. While this suggests the presence of a single virus circulating in Greece at that time, the 9.8% difference between the sequences of viruses recovered in Greece and in Germany (Figure 6) indicate the existence of distinct strains of HAV in Northern and Southern Europe during 1982-1983. Of particular interest, however, was the fact that a fecal sample (LV-BE) collected from an American soldier involved in an epidemic of hepatitis A at a military prison in Kansas, USA during 1982 contained virus with a sequence which was identical to that of the virus causing the Grafenwohr disease outbreak in the Federal Republic of Germany (see Lednar et al., 1985, and relevant EPICON reports for descriptions of these hepatitis A outbreaks). These sequence data, obtained directly from viruses present in feces, establish a clear but previously unrecognized epidemiologic link

between these two military epidemics, and demonstrate the power of this molecular approach to epidemiology and disease surveillance activities.

While most human HAV strains were closely related, H122 and CF53 were strikingly different (Figure 6). These viruses, recovered from patients in Sweden and France during 1986 and 1987 respectively, differ from each other and the remaining human HAV strains at 16-24% of base positions sequenced (a degree of difference approaching that existing between different serotypes of poliovirus). The H122 virus, however, shared 97% nucleotide identity with PA21 virus, which was recovered from a captive owl monkey and has been considered to be of simian origin (Brown et al., 1989). Although H122 and CF53 strains show considerable genetic divergence, they were detected by AC/PCR employing an HAV-specific monoclonal antibody and thus appear to be closely related serologically to other human strains. The biological significance of these newly recognized HAV genotypes remains uncertain and will require further investigation. The genetic divergence manifested by these strains cannot be related to geographic site or time of collection, to disease expression, or to risk factors for acquisition of hepatitis A given presently available data.

CONCLUSIONS

PEPSCAN work continues to confirm the highly conformational nature of the HAV antigenic site. It is surprising, however, that a greater degree of reactivity with short oligopeptides has not been demonstrated, given previous PEPSCAN studies with FMDV outside of its major (sequential) antigenic loop and studies of the immunogenicity of poliovirus and rhinovirus peptides. These studies remain in progress, however, and it is our intention to complete PEPSCAN analysis with a panel of over 20 neutralizing monoclonal antibodies available for study.

We are encouraged by the results obtained thus far with the poliovirus/HAV chimeras. The presentation of HAV peptide sequences within the βB-βC loop of Sabin type 1 poliovirus appears to place important conformational constraints upon the peptide that may favorably influence its immunogenicity. This seems to be the case with the chimeras S1/H2 and S1/H15, as peptide constructs similar to the HAV sequences included in these chimeras, conjugated to keyhole limpet hemocyanin (KLH) as a protein carrier, have failed to elicit anti-HAV neutralizing activity. These serologic results suggest that chimeric picornaviruses may have promise as possible HAV vaccines, and are also useful tools for further dissection of the functional antigenic structure of HAV.

The S1/H15 chimera is particularly interesting, as its ability to induce a neutralizing response in some animals suggests the existence of an accessory neutralization domain near the amino terminus of HAV VP1. The data suggest that the HAV residues expressed by S1/H15 exist in a conformation that is distinct from that of the related peptide 639c (HAV residues 1011-1032) and which more closely mimics the conformation assumed by the amino terminal domain within the virus structure. However, the chimera appears to present the HAV sequence in at least two conformations. The predominant conformation appears to be one which induces an anti-peptide response and is a target for chimera neutralization and

precipitation by anti-peptide antisera, but does not mimic the native HAV epitope. The second, apparently minor conformation is that which is capable of inducing anti-HAV neutralizing activitiy.

Two approaches are being taken to further evaluate these findings. First, in collaboration with Dr. M. Ferguson at NIBSC, an effort is underway to select S1/H15-specific monoclonal antibodies with anti-HAV neutralizing activity. In addition, octapeptides representative of the βB-βC loops of Sabin type 1 and the chimeric S1/H15 virus (PV1 residues 1087-1108), as well as the cognate amino terminal domain of HAV VP1 (residues 1006-1034) have been recently synthesized for use in PEPSCAN analysis of anti-HAV polyclonal, anti-chimera and anti-peptide sera. It is hoped that PEPSCAN studies may allow "fine-tuning" of the epitope inserted into the chimeric virus. In addition, as heat denaturation of poliovirus markedly affects its antigenicity, we will examine the immunogenicity of heat-denatured chimeras S1/H15 and S1/H2 ("H" particles).

We report continued progress in construction of an infectious cDNA clone derived from a virulent HAV variant. The lethal defect in the p16 full-length clone is within the region bounded by bases 4977 and 7003. This region is being resequenced, and new clones are being generated in which this region has been replaced by cDNA from p16 and wt viruses. We anticipate that these efforts will result in an infectious clone from which virulent HAV may be recovered. Such a clone will allow the exploration of new approaches to attenuation of HAV, including the manipulation of the 5′ NTR. Our analysis of viruses containing chimeric 5′ NTRs derived in part from virulent and attenuated HAVs has indicated an important role for the 5′ NTR in controlling replication in cell culture (Day et al., 1990b). Based in part on recent studies with poliovirus (Iizuka et al., 1989), we suspect that viable 5′ NTR deletion mutants of HAV will be significantly attenuated in primates.

Sequence data generated from 47 HAV strains using the AC/PCR approach will be useful in analysis of HAV strains isolated during future HAV vaccine trials and in future disease surveillance activities. We have recently initiated a collaboration with B. Robertson of the Centers for Disease Control in an effort to extend these studies to other available HAV strains. We also plan detailed antigenic analysis of CF53 and H122 viruses which are representative of new HAV genotypes. In addition to its value in providing new information about the molecular epidemiology of HAV, the AC/PCR technique should be applicable to other surevillance studies of other militarily relevant virus infections, including enterically transmitted non-A hepatitis viruses, such as hepatitis E virus (HEV).

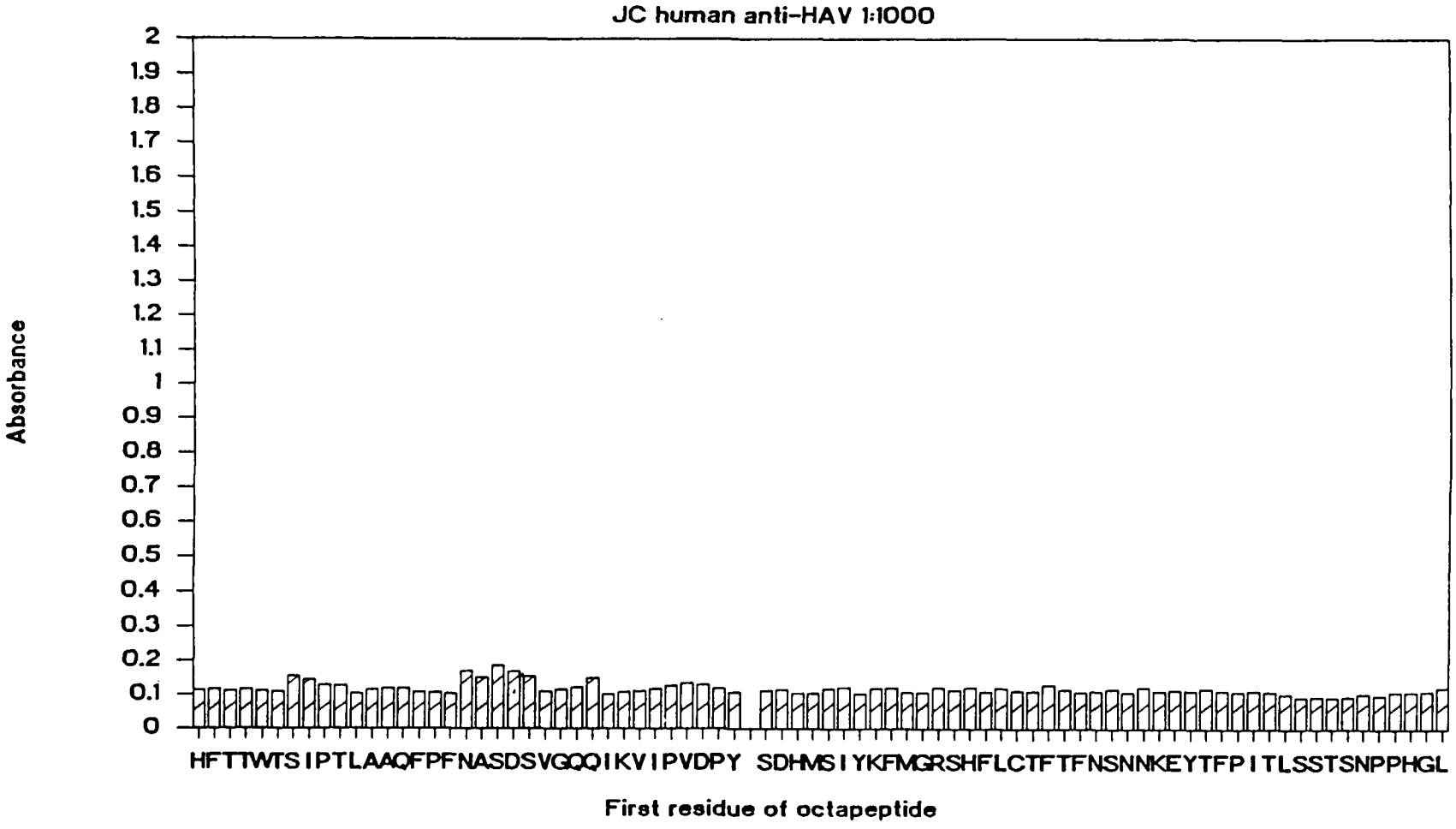

Figure 1. Oligopeptide scan of the probable βB-βC loops of VP3 (left hand block, residues 3050-3091) and VP1 (right hand block, residues 1080-1130) of HM175 strain HAV, with a high-titered human polyclonal anti-HAV positive serum (JC).

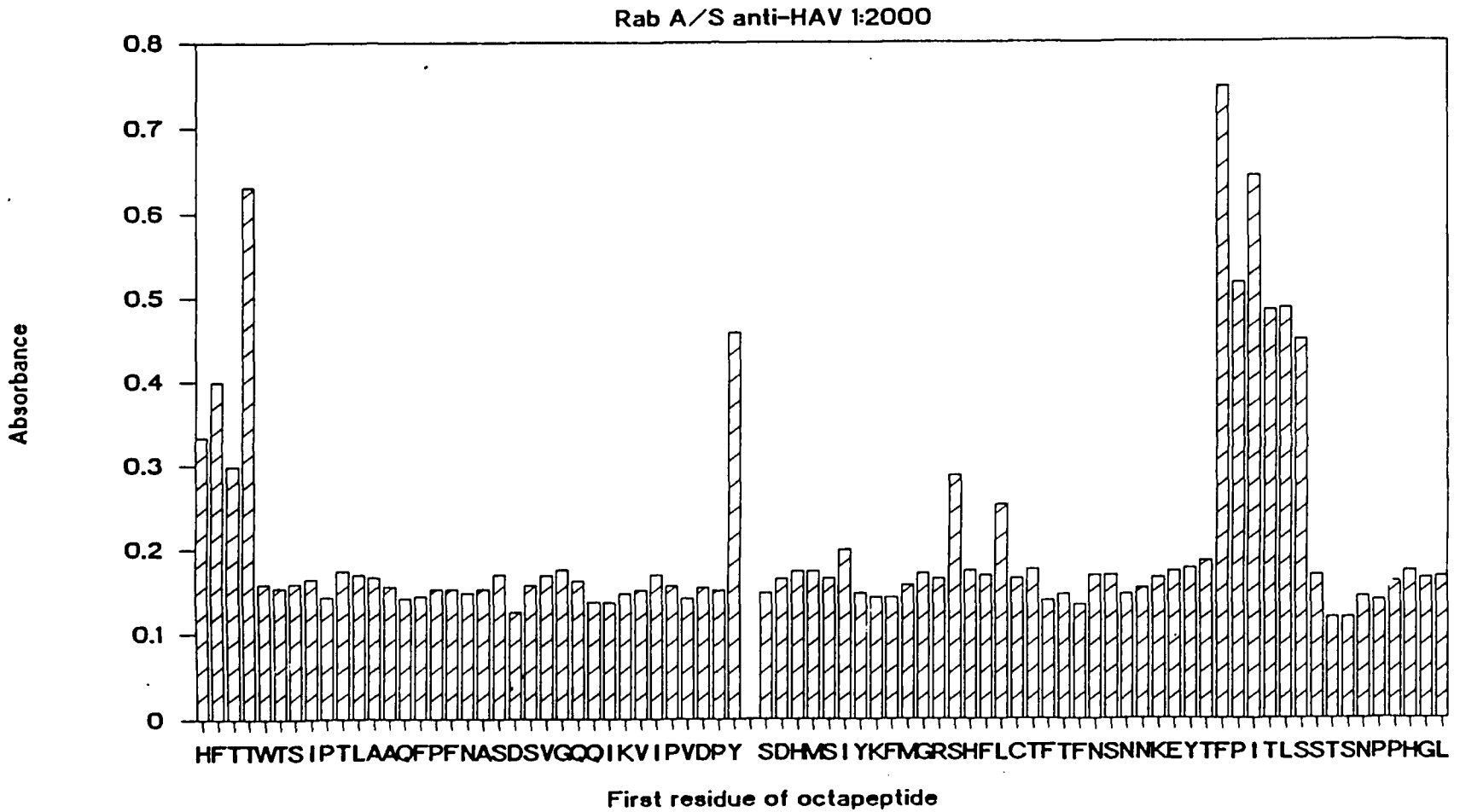

Figure 2. Oligopeptide scan of the probable βB-βC loops of VP3 (left hand block, residues 3050-3091) and VP1 (right hand block, residues 1080-1130) of HM175 strain HAV, with rabbit hyperimmune anti-HAV serum A/S.

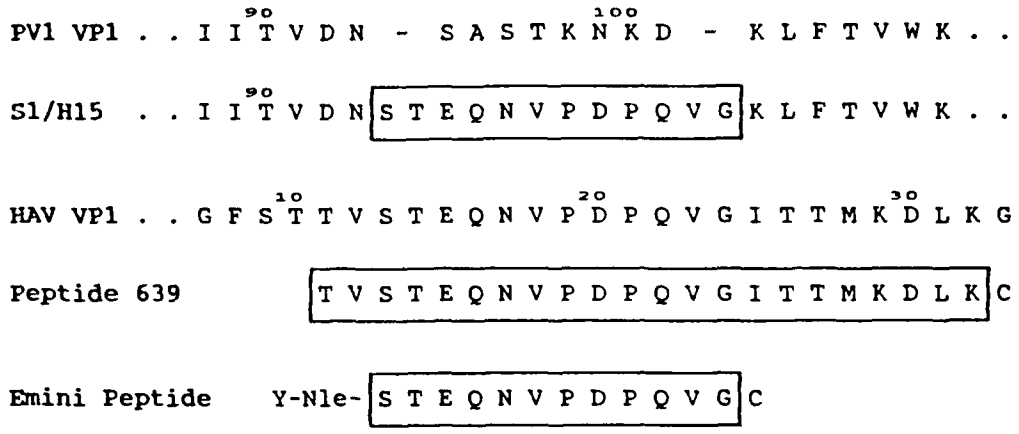

Figure 3. Peptide sequences of VP1 βB-βC loop of Sabin type 1 poliovirus (top), HAV/poliovirus chimera S1/H15, amino terminal domain of VP1 of HAV, and related peptides. Peptide 639 and the Emini peptide (Emini et al., 1985) were conjugated to carrier proteins through carboxy terminal cysteine residues.

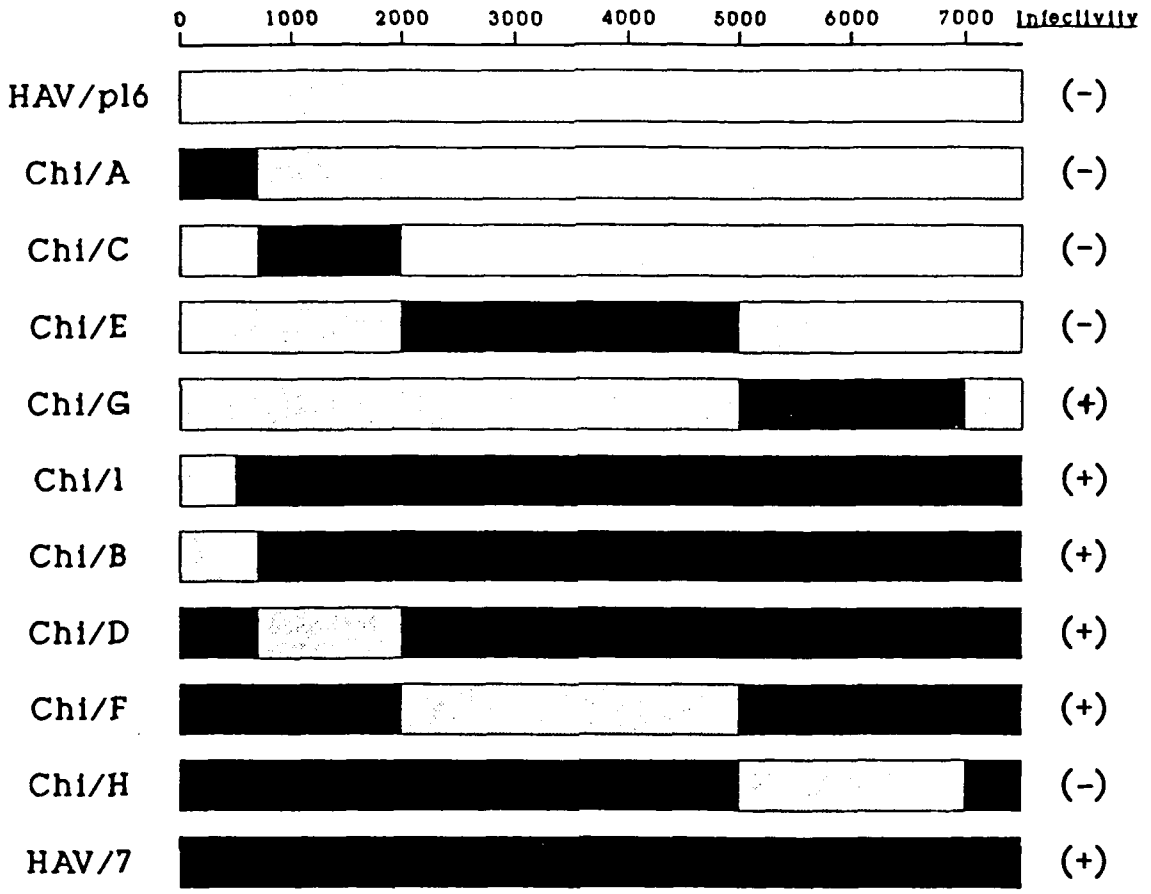

Figure 4. Chimeric genomic-length cDNA constructs containing regions derived from cell culture-adapted but virulent p16 HM175 (stippled regions), and cell culture-adapted, attenuated p35 HM175 virus (pHAV/7 cDNA, shaded regions). Whether or not infectious virus was produced following transfection of BS-C-1 cells with related RNA transcripts is indicated to the right.

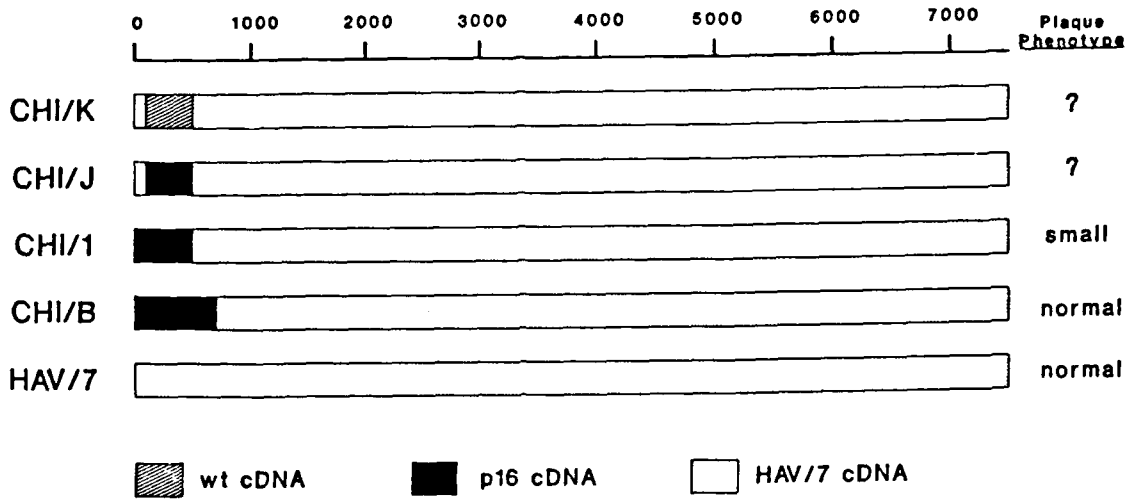

Figure 5. Genomic maps of 5' NTR HAV chimeras. The background virus (stippled) is p35 HM175 (pHAV/7). Segments of the 5' NTR have been replaced with related regions from p16 HM175 (darkly shaded) or wt HM175 (cross hatched) virus genomes. The "plaque"-morphology of virus derived from each clone, as assessed in radioimmunofocus assays, is shown at the right. Chi/J and Chi/K are currently under evaluation.

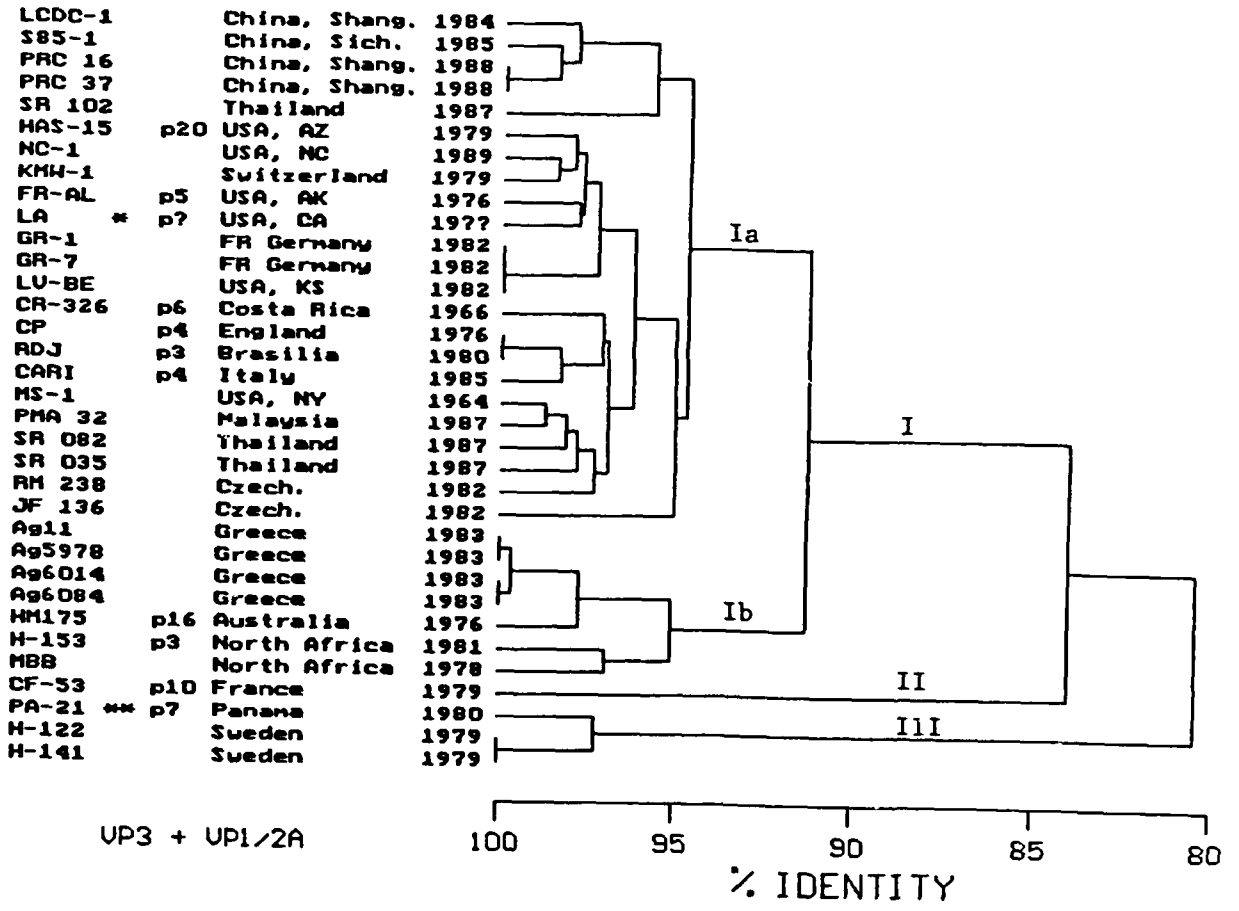

Figure 6. Dendrogram constructed by comparative subset averaging of percent nucleotide identity data derived by AC/PCR from HAV strains representing epidemiologically diverse sources. The geographic location and date of collection of each strain is noted, as is the passage level in cell culture for those sequences derived from cell culture-adapted viruses. The approximate degree of nucleotide identity between any two strains is represented by the distance from the left of the diagram to the first common node. See text for details.

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